DEMONSTRATION OF FLAVONOID ENZYMES IN CYANIC AND ACYANIC PELARGONIUM-ZONALE-HYBRIDS

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Abstract

The cyanic and acyanic colour of Pelargonium cultivars is the result of the presence or absence of anthocyanins in the flowers. Instead of anthocyanins, acyanic flowers contain different types and amounts of flavonoids leading to white or cream colour. Our aim was to demonstrate the enzymes of the anthocyanin pathway and to identify possible blocks in the main enzymatic steps. We investigated four enzymes, chalcone synthase (CHS), flavanone 3β-hydroxylase (FHT), dihydroflavonol 4-reductase (DFR) and flavonol synthase (FLS). The first three enzymes lead to the production of leucoanthocyanidins, the substrates of anthocyanidin synthase (ANS), and FLS is responsible for flavonol synthesis. In spite of many examples of testing the above enzymes in numerous species, up to now, Pelargonium proved to be a recalcitrant plant for enzymatic studies. Nevertheless, we detected significant differences in enzyme activity between cultivars and between flower developmental stages of the same cultivar. The acyanic cultivars studied seem to be blocked late in the anthocyanin pathway. Our results may serve as a basis for further genetic and biochemical characterisation of Pelargonium plants.

1. Introduction

The colour of Pelargonium cultivars solely derives from the presence or absence of flavonoid and anthocyanin pigments and depends on their quality and quantity as reported by Asen and Griesbach (1983) and Mitchell et al. (1998). The expression of their full colour range is restricted by the low pH of the vacuoles. Carotenoid based colours are absent from Pelargonium-zonale-hybrids and Pelargonium-peltatum-hybrids, therefore the available spectrum is quite narrow. The acyanic flowers contain different types and amounts of flavonoids leading to white or cream colour. Detailed knowledge about the biosynthetic potential of these lines can help us to extend the range of naturally occurring colours in this species. Beyond and following the biochemical characterisation we investigated the activity and specificity of four enzymes of the flavonoid pathway (Fig. 1). These enzymes were: chalcone synthase (CHS) (The CHS preparation also contained active chalcone isomerase CHI.), flavanone 3β-hydroxylase (FHT), dihydroflavonol 4-reductase (DFR) and flavonol synthase (FLS). They can be rate limiting or end-product determinative factors in flavonoid biosynthesis. The first three enzymes lead to the production of leucoanthocyanidins, the substrates of anthocyanidin synthase (ANS), and FLS shares substrate with DFR and catalyses the synthesis of flavonols leading to cream colour if highly accumulated. Presence of secondary metabolites, like hydrolysable tannins, in Pelargonium flowers strongly interferes with the extraction of active enzymes.
2. Materials and methods

2.1. Plant material

Commercial varieties and breeding lines of Elsner pac® Jungpflanzen Dresden were used in this study. They were grown under identical, standard greenhouse conditions. For the different cultivars comparison of enzyme activities were carried out at the same developmental stages.

2.2. Chemicals

$^{14}$C labelled malonyl-CoA was purchased from the American Radiolabelled Chemicals INC. 4-coumaroyl-CoA was kind gift from W. Heller. Labelled naringenin, dihydrokaempferol and dihydroquercetin were prepared according to the methods described by Britsch et al. (1981, 1992). Authentic flavonoids were from our laboratory collection.

2.3. Enzyme preparations and enzyme assays

Enzyme extractions and reaction conditions were according to the methods of Claudot et al. (1992) and Dellus et al. (1997). Labelled flavonoids were used as substrates in the tests with $3 \times 10^5$ Bq activity (5000 disintegrations per minute - dpm), naringenin for FHT, dihydrokaempferol and dihydroquercetin for both DFR and FLS. For CHS $66 \times 10^5$ Bq (11000 dpm) $^{14}$C malonyl-CoA and unlabelled 4-coumaroyl-CoA served as substrates. In each reaction 20 µl of enzyme in 200 µl assay volume was incubated for 30 minutes at 30°C. Enzyme tests were terminated and extracted by the addition of 2x 100µl ethyl acetate.

2.4. Analytical methods

The organic phase, containing the reaction products and the rest of the flavonoid substrates, was separated by centrifugation and chromatographed on cellulose TLC plates (Merck, Nr. 105716) in CAW (chloroform : acetic acid : water 50 : 45: 5) for CHS, FHT, and DFR. FLS tests were separated in 30% acetic acid. The air dried TLC plates were imaged, quantified and evaluated using FUJI BAS Reader 1000 and Tina 2.09e image analyser software (raytest, Isotopenmessgeräte GmbH, Germany). Products were identified by their Rf values and by co-chromatography with authentic compounds.

2.5. Measurement of colour intensity

We measured the colour of selected cultivars with the Minolta Chroma Meter using the CR-300 measuring head (Minolta, Japan) in the L* a* b* colour system. For our comparison we used the colour intensity as the reciprocity ($1/L^*$) of the lightness variable.

3. Results

3.1. Chalcone synthase and flavanone 3ß-hydroxylase

In ‘Robe’, a dark red flowering cultivar, we examined the activity of CHS in detail. Characteristic alteration of enzyme activity was found during flower development with the maximum at stage 4 (Fig. 2 and Fig. 3). The activity of CHS, the key enzyme of the flavonoid pathway, correlated with the intensity of flower colouration in the different cultivars (Fig. 4). The correlation was similar for FHT, except for ‘Alba’, a white cultivar, which has shown comparably higher activity (Fig. 4). The activity of CHS and

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FHT was demonstrated in all examined lines.

3.2. Dihydroflavonol 4-reductase

DFR activity was only demonstrable in cyanic cultivars whereas with the methods used it was not detectable in the examined white cultivars (data not shown). We demonstrated a difference in substrate specificity of DFR in cyanic cultivars belonging to pelargonidin or cyanidin/delphinidin colour types. ‘Avenida’ and ‘Perlenkette Orange’ two pelargonidin accumulating lines accepted dihydrokaempferol to a similar extent as dihydroquercetin, whereas ‘Penve’ and ‘Meloblue’ – cyanidin and delphinidin types, respectively, both lacking pelargonidin – hardly accepted dihydrokaempferol as substrate but readily converted dihydroquercetin to leucocyanidin (Fig. 5). Dihydromyricetin, the delphinidin precursor, was not tested.

3.3. Flavonol synthase

In the cream breeding line ‘BS’ flavonol synthase was detected, dihydrokaempferol and dihydroquercetin served as substrate for the synthesis of the flavonols, kaempferol and quercetin, respectively (Fig. 6). Although in ‘BS’ FLS was present over many bud developmental stages, in cyanic cultivars activity was only found in buds of early developmental stages (data not shown).

4. Discussion

The petals of Pelargonium flowers contain high amounts of hydrolysable tannins, which hindered the enzymatic investigation of flavonoid biosynthesis. The use of special protective buffers, described by Claudot et al. (1992) and Dellus et al. (1997), was necessary to extract these enzymes in an active form. We have examined the flavonoid and anthocyanin biosynthesis in selected breeding lines and varieties. We observed differences between cultivars and between different stages of bud and flower development within the same cultivar.

The cyanic and acyanic lines contained CHS and FHT activity to an extent which correlates with the colour intensity of their flowers. None of the examined acyanic lines turned out to be mutant for these enzymatic steps. This is in accordance with the biochemical results of Horn (1994), who has found accumulation of dihydroflavonols in all acyanic cultivars.

Dihydroflavonols, like dihydrokaempferol, dihydroquercetin and dihydromyricetin are converted to the respective leucoanthocyanidins by DFR. In our study DFR activity was detected in cyanic lines with characteristic substrate specificities corresponding to the main anthocyanidin type present in their flowers. Thus, the substrate specificity of DFR is an important control point of anthocyanin formation. It determines the type of anthocyanidin accumulating in the flower from the available precursors. In acyanic lines we failed to detect DFR activity. This can be due to very low activities or mutation in this enzymatic step. DFR mutant lines leading to acyanic colour have already been identified in Callistephus chinensis, Matthiola incana, Petunia hybrida and other species as reviewed by Forkmann and Heller (1999).

Dihydroflavonols are substrates of flavonol synthase as well. FLS activity leads to the formation of flavonols: kaempferol, quercetin and myricetin, which can be further modified by glycosylation. Flavonol glycosides are copigments in cyanic flowers and can lead to cream colour in acyanic ones. We successfully detected FLS activity in most flower stages of the cream coloured breeding line ‘BS’. In accordance with the long lasting activity and the substrate specificity of FLS, high amounts of kaempferol and quercetin glycosides are accumulated in the flowers of this line. Although flavonols are also present in the flowers of cyanic Pelargonium lines, the corresponding enzyme activity was only detected in early bud stages, whereas in white lines FLS activity was
not detected.

We conclude that the lack of anthocyanin synthesis is most probably the consequence of the absence, or highly reduced activity of DFR in the examined acyanic lines. This might be accompanied by the lack of subsequent enzymes in the pathway as well. Characterisation of additional flavonoid specific enzymes is under way in this species.

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References


Figure 1. Schematic pathway of flavonoid biosynthesis.

**Abbreviations:** CHS – chalcone synthase; CHI – chalcone isomerase; FHT – flavanone 3β-hydroxylase; FLS – flavonol synthase; DFR – dihydroflavonol 4-reductase; ANS – anthocyanidin synthase; UFGT – UDP-glucose: flavonoid 3-O-glucosyltransferase; THC – 2’,4,4’,6’-tetrahydroxychalcone; Nar – naringenin; DHQ – dihydroquercetin; DHK – dihydrokaempferol; DHM – dihydromyricetin; LCy – leucocyanidin; LPg – leucopelargonidin; LDp – leucodelphinidin; Qu – quercetin; Km – kaempferol; My – myricetin; Cy – cyanidin; Pg – pelargonidin; Dp – delphinidin.
Figure 2. Characteristic bud stages of *Pelargonium-zonale-hybrids*. Colouration starts at the inner side of petals at stage 3.

Figure 3. Course of CHS enzyme activity in the red cultivar ‘Robe’ during flower development.
Figure 4. Correlation of CHS and FHT activity and intensity of flower colouration. FHT activity is shown as conversion % of naringenin to dihydrokaempferol; CHS activity is shown in comparison to ‘Robe’ (‘Robe’=100%); 1/L* is the reciprocity of the lightness of the colour.

Figure 5. Comparison of substrate specificity of DFR. Pelargonidin types (‘Avenida’ and ‘Perlenkette Orange’) convert dihydrokaempferol (DHK) to a higher extent, than delphinidin/cyanidin types (‘Meloblue’ and ‘Penve’). The result is expressed as the % of radioactivity measured in the product, leucoanthocyaidin.
Figure 6. Presence of DFR and FLS activity in breeding lines. Dihydroflavonols (DHK and DHQ) serve as substrate for both enzymes. The result is expressed as the % of radioactivity measured in the product after enzyme tests. DFR activity was not detectable in ‘BS’ and FLS in ‘Avenida’ respectively.